

## FINAL REPORT

**Test Facility Study No. 511883**

### **96-Hour Acute Toxicity Study in Carp with MLA-3202 (Semi-Static)**

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**03 April 2017**

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## 1. STATEMENT OF GLP COMPLIANCE

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands

All phases of this study performed by the test facility were conducted in compliance with:

- OECD Principles of Good Laboratory Practice;
- EC Council Directive 2004 (2004/10/EC, February 11, 2004, Official Journal of February 20, 2004).

The data generated and reported are considered to be valid.

Charles River Den Bosch

Signature:



Name: M.A. Tobor-Kaplon, PhD.

Title: Study Director

Date: 03 April 2017

## 2. TEST FACILITY QUALITY ASSURANCE STATEMENT

Charles River Den Bosch, 's-Hertogenbosch, The Netherlands.

Study title: 96-Hour acute toxicity study in carp with MLA-3202 (semi-static)

This report was inspected by the Charles River Den Bosch Quality Assurance Unit (QAU) according to the Standard Operating Procedure(s).

The reported method and procedures were found to describe those used and the report reflects the raw data.

During the on-site process inspections, procedures applicable to this type of study were inspected.

The dates of Quality Assurance inspections are given below.

Project	511883			
Type of Inspections	Phase/Process	Start Inspection date	End Inspection date	Reporting date to TFM and SD*
Study	Study Plan	10-Aug-2016	10-Aug-2016	10-Aug-2016
	Study Plan Amendment 01	27-Oct-2016	27-Oct-2016	27-Oct-2016
	Study Plan Amendment 02	06-Jan-2017	06-Jan-2017	06-Jan-2017
	Report	09-Mar-2017	09-Mar-2017	09-Mar-2017
Process	<b>Test Substance Receipt</b>	21-Nov-2016	21-Nov-2016	21-Nov-2016
	Test Substance Handling			
<b>Analytical and physical chemistry</b>		01-Dec-2016	28-Dec-2016	28-Dec-2016
Test Substance Handling				
Exposure				
Observations/Measurements				
Specimen Handling				
<b>Environmental Science</b>		09-Jan-2017	23-Jan-2017	23-Jan-2017
Test Substance Handling				
Exposure				
Observations/Measurements				

\*TFM=Test Facility Management SD = Study Director

The facility inspection program is conducted in accordance with Standard Operating Procedure.

The review of the final report was completed on the date of signing this QA statement.

Charles River Den Bosch

Signature: .....



Name: **Bart Kluskens, BSc**  
**Quality Assurance Auditor**

Date: .....



### 3. SUMMARY

96-Hour Acute Toxicity Study in Carp with MLA-3202 (semi-static).

The study procedure described in this report was based on the OECD guideline No. 203, 1992. In addition, the procedures were designed to meet the test methods of the Commission Regulation (EC) No 440/2008, Part C.1, 2008 and the OECD series on testing and assessment number 23, 2000.

The batch of MLA-3202 tested was a clear amber-red liquid completely soluble in test medium at the concentrations tested. The test item was a UVCB substance. No correction was made for the purity/composition of the test item.

A final test was performed based on results of a combined limit/range-finding test. Seven fish per group were exposed to an untreated control and nominal concentrations of 0.22, 0.46, 1.0, 2.2 and 4.6 mg/L. The total exposure period was 96 hours. Test solutions were renewed daily and samples for analytical determination of exposure concentrations were taken at the start and the end of the first and the last renewal.

Samples taken from all concentrations were analysed. The actual concentrations measured in the freshly prepared solutions were in agreement with nominal (89-100%). Concentrations measured in the spent solutions at the end of the first refreshment period were between 44 and 90% of initial. The stability of the concentrations was increasing with the nominal dose. The actual concentrations in the spent solutions measured at the end of the last renewal period were at the level of 72-84% of initial.

Based on these results, the average exposure concentrations were 0.16, 0.33, 0.85, 1.8 and 4.0 mg/L in nominally of 0.22, 0.46, 1.0, 2.2 and 4.6 mg/L, respectively.

The study met the acceptability criteria prescribed by the study plan and was considered valid.

Under the conditions of the present study, the 96h-LC<sub>50</sub> was 0.91 mg/L based on average exposure concentrations (95% confidence interval between 0.69 and 1.2 mg/L).

## 4. INTRODUCTION

### 4.1. Study schedule

Experimental starting date : 21 November 2016  
Experimental completion date : 26 January 2017

### 4.2. Purpose

The purpose of the study was to evaluate the test item for its ability to generate acute toxic effects in *Cyprinus carpio* during an exposure period of 96 hours and, if possible, to determine the LC<sub>50</sub> at all observation times.

### 4.3. Guidelines

The study procedure described in this report is in compliance with the OECD guidelines for Testing of Chemicals, guideline No. 203: "Fish Acute Toxicity Test", Adopted 17 July, 1992.

In addition, the procedure is designed to meet the test methods prescribed by the following guideline and guidance documents:

- Council Regulation (EC) No 440/2008 of 30 May 2008, Part C: Methods for the determination of ecotoxicity, Publication No. L142, C.1. "Acute Toxicity for Fish".
- Guidance document on aquatic toxicity testing of difficult substances and mixtures, OECD series on testing and assessment number 23, 2000.

### 4.4. Retention of records and materials

Records and material pertaining to the study, which include study plan and amendments, raw data, specimens, except perishable specimens, and the final report will be retained in the archives of the test facility for a minimum of 5 years after the finalization of the report. After this period, the sponsor will be contacted to determine how the records and materials should be handled. The test facility will retain information concerning decisions made.

Perishable specimens (e.g. requiring refrigeration or freezing) will be discarded following evaluation in the study without further notice to the study sponsor.

A sample of the test item will be retained until expiry date or applicable retest date. After this period the sample(s) will be destroyed.

### 4.5. Responsible personnel

#### 4.5.1. Test facility

Study Director M.A. Tobor-Kaplon, PhD.

Principal Scientist (Analytical Chemistry) M.J.C. Brekelmans, MSc.

#### 4.5.2. Sponsor Representative

Study Monitor Audrey Batoon, PhD.

### 4.6. Definitions

Fish were considered to be **dead** when no reaction was observed after touching the caudal peduncle and visible breathing movements were absent. In addition, fish that were convulsing or showing other severe forms of distress not considered transient in nature and likely to become more severe before the exposure is terminated, will be sacrificed for humane reasons. These fish will be treated as having died in the test, based on OECD guideline on humane endpoint (ENV/JM/MONO/(2000)7).

The LC<sub>50</sub> is the concentration killing 50% of the fish after a defined period of exposure.

## 5. MATERIALS AND METHODS

### 5.1. Test item

#### 5.1.1. Test item information

Test item	207258/A
Identification	MLA-3202
Appearance	Clear amber-red liquid
Batch	RC-1045
Purity/Composition	UVCB
Test item storage	At room temperature
Stable under storage conditions until	17 February 2019 (expiry date)
See <a href="#">APPENDIX 3</a> ; Certificate of analysis.	

#### 5.1.2. Study specific test item information

Purity/composition correction factor	No correction factor required
Chemical name (IUPAC), synonym or trade name	Amides, tallow, N,N-bis(2-hydroxypropyl)
CAS Number	1454803-04-3

### 5.2. Vehicle information

Solubility in water:	< 1 g/L
Stability in water:	Yes

### 5.3. Reference item

This report includes the results of a reference test with pentachlorophenol (PCP) ([APPENDIX 2](#)).

### 5.4. Preparation of the test solution

The batch of MLA-3202 tested was a clear amber-red liquid completely soluble in test medium at the concentrations tested. The test item was a UVCB substance. No correction was made for the purity/composition of the test item.

Preparation of test solutions started with the highest concentration of 4.6 mg/L applying approximately one hour of magnetic stirring to accelerate the dissolution of the test item in the test medium. The lower test concentrations were prepared by subsequent dilutions of the highest concentration in test medium. All final test solutions were clear and colourless.

It should be noted that in the range-finding test the highest test concentration was 100 mg/L and was stirred for a period of 2.5 hours. The highest concentration in the range-finding test was hazy, while other concentrations were clear and colourless.

### 5.5. Test system

Species	Carp ( <i>Cyprinus carpio</i> , Teleostei, Cyprinidae) Linnaeus, 1758
Source	Zodiac, proefacc, "De Haar Vissen", Wageningen University and Research Centre, The Netherlands.
Mean length <sup>1</sup>	Range-finding test: 3.3 ± 0.2 cm Final test: 3.0 ± 0.3 cm

<sup>1</sup> Ten fish of the batch used for the test, were weighed and measured prior to the start of the test.

Mean weight <sup>1</sup>	Range-finding test: $1.1 \pm 0.18$ g Final test: $0.36 \pm 0.13$ g
Characteristics	F1 from a single parent-pair bred in UV-treated water.
Reason for selection	This system has been selected as an internationally accepted species.
Total fish used	54
<b>5.6. Holding</b>	
Quarantine/Acclimatisation	At least 12 days after delivery.
Medium	Adjusted ISO medium, formulated using RO-water (tap-water purified by reverse osmosis; GEON Waterbehandeling, Berkel-Enschot, The Netherlands) with the following composition:  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 211.5 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 88.8 mg/L $\text{NaHCO}_3$ 46.7 mg/L $\text{KCl}$ 4.2 mg/L
Measurements	Conductivity, pH, nitrate, nitrite and ammonia concentration: once a week. Temperature: continuous. In addition, pH and temperature were measured before transferring the fish to the test system.
Water quality parameters	Were kept within the optimum limits for the respective fish species.
Feeding	Daily with pelleted fish food (Cyprico Crumble Excellent (300-500 um), Coppens International bv, Helmond, The Netherlands)
Validity of batch	In the batch of fish used for the test, mortality during the seven days prior to the start of the test was less than 5%.

### **5.7. Range-finding test**

A range-finding test was performed to provide information about the range of concentrations to be used in the final test. Test procedure and conditions were similar to those applied in the final test with the following exceptions:

- Three fish per concentration were exposed to a range of 0.10 to 100 mg/L increasing by a factor of 10.
- Dissolved oxygen concentrations, pH and temperature were only measured in the lowest and the highest test concentration besides day 1.
- Test was performed without renewal of test solutions.

**5.8. Final test****5.8.1. Test concentrations**

MLA-3202                    0.22, 0.46, 1.0, 2.2 and 4.6 mg/L  
Control                        Test medium without test item or other additives

**5.8.2. Test procedure and conditions**

Test duration                96 hours  
Test type                    Semi-static, with daily renewal of test solutions  
Test vessels                6.5 litres, all-glass, containing 5 litres of test solution  
Test medium                Adjusted ISO medium with a hardness of 180 mg CaCO<sub>3</sub> per litre and a pH of 7.7 ± 0.3.  
Number of fish              7 fish per concentration and control  
Loading                     0.50 g fish/Litre, i.e. 7 fish per 5 litres of test medium  
Illumination                16 hours photoperiod daily  
Aeration                    The test media were not aerated during the test.  
Feeding                    No feeding from 24 hours prior to the test and during the total test period.  
Introduction of fish        Within 26 minutes after preparation of the test media from a holding tank with comparable water quality parameters and pH and temperature differences between test and holding tank media of less than 0.5 unit and 1.0°C.  
Euthanasia                  At the end of the test the surviving fish were rapidly killed by exposing them to ca. 1.2% ethylene glycol monophenylether in water.

**5.8.3. Sampling for analysis of test concentrations**

During the final, test samples for possible analysis were taken from all test concentrations and the control according to the schedule below. The method of analysis is described in the appended Analytical Report ([APPENDIX 4](#)).

Frequency                    1. At the start of the test and after 72 hours from the freshly prepared solutions.  
                              2. At the first renewal (t=24h) and the end of the test from the 24-hour old solutions.  
  
                              In addition samples were taken at nominal days 2 and 3 from spent solutions of groups where total mortality was observed.  
Volume                      1.0 mL from the approximate centre of the test vessels  
Storage                     Samples were stored in a freezer until analysis.

Additionally, single reserve samples of 1.0 mL were taken from all test solutions for possible analysis. If not already used, these samples were stored in a freezer for a maximum of three months after delivery of the draft report, pending on the decision of the sponsor for additional analysis.

#### 5.8.4. Measurements and recordings

Mortality and other effects	At 2¾, 24, 48, 72 and 96 hours following the start of exposure. In addition, every morning from day 1 to observe for any dead or severely distressed fish. Dead fish were removed when observed.
Dissolved oxygen content, pH and temperature	Daily in all vessels with surviving fish, beginning at the start of the test (day 0).

### 5.9. Interpretation

#### 5.9.1. Data handling

Determination of the average exposure concentrations:

$$\text{The average exposure concentrations were calculated as } \frac{\sqrt{C_{t=0} \times C_{t=24,\text{old}}} + \sqrt{C_{t=72,\text{fresh}} \times C_{t=96}}}{2}$$

being the mathematical means of the concentrations of MLA-3202 measured in the samples taken during the first and the last refreshment period for the nominal concentrations of 0.22, 0.46 and 1.0 mg/L. For the remaining concentrations it was calculated as  $\sqrt{C_{t=0} + C_{t=24,\text{old}}}$ , because no fish survived in these concentrations until the end of the exposure.

The LC<sub>50</sub> was determined using the maximum likelihood estimation method with the weibits of the percentages of dead fish as function of the logarithms of the corresponding concentrations (Weibull analysis).

No 24h-LC<sub>50</sub> could be calculated because the responses were below 50%.

The calculations were performed with ToxRat Professional v.3.2.1. (ToxRat Solutions®, GmbH, Germany).

#### 5.9.2. Acceptability of the test

1. No mortality was observed in the control at the end of the test.
2. Test conditions were maintained constant throughout the test.
3. The dissolved oxygen concentration has been at least 60% of the air saturation value throughout the test (>5 mg/L at 22°C).
4. Since measured concentrations deviated by more than 20% from initially measured, results were based on the average exposure concentration.

### 5.10. List of deviations

#### 5.10.1. List of study plan deviations

There were no deviations from the study plan.

#### 5.10.2. List of standard operating procedures deviations

Any deviations from standard operating procedures were evaluated and filed in the study file. There were no deviations from standard operating procedures that affected the integrity of the study.

## 6. ELECTRONIC SYSTEMS FOR DATA ACQUISITION

The following electronic systems were used for data acquisition:

- REES Centron Environmental Monitoring system version SQL 2.0 (REES Scientific, Trenton, NJ, USA).

## 7. RESULTS

### 7.1. Range-finding test

The results of the range-finding test are presented in [Table 1](#). No mortality or clinical effects were observed in the lowest concentration tested during the entire exposure period. Fish exposed to the two highest concentrations did not survive the first 24 hours of exposure. All fish exposed to 1.0 mg/L were observed to be hypoactive at 48 hour of exposure and immobilised from the 72 hour onwards. The expected LC<sub>50</sub> values were between 1.0 and 10 mg/L.

Based on these results samples taken from nominal concentrations of 0.10, 1.0 and 10 mg/L were analysed. The initial concentrations were at the level of nominal (102 -112%). The concentrations measured in 10 mg/L remained stable (98-113% of initial) during the exposure period. At the lower concentrations, the measured concentration decreased substantially; i.e. below the limit of detection in the lowest group and to 0.34% of initial in nominally 1.0 mg/L (see also [Table 2](#) of the appended Analytical Report). Based on these results, it was decided, to perform the full test with a daily renewal of test solutions.

All test conditions were maintained within the limits prescribed by the study plan.

**Table 1**  
**Incidence of mortality and total mortality during the range-finding test**

MLA-3202 Nominal concentration (mg/L)	Initial number of fish	Cumulative mortality					Total Mortality (%)
		3¾h	24h	48h	72h	96h	
0.1	3	0	0	0	0	0	0
1.0	3	0	0	0	0	0	0
10	3	0	3	3	3	3	100
100	3	0	3	3	3	3	100

### 7.2. Final test

#### 7.2.1. Measured concentrations

The results of analysis of the samples taken during the final test are described in [Table 3](#) of the appended Analytical Report.

Samples taken from all concentrations were analysed. The actual concentrations measured in the freshly prepared solutions were in agreement with nominal (89-100%). Concentrations measured in the spent solutions at the end of the first refreshment period were between 44 and 90% of initial. The stability of the concentrations was increasing with the nominal dose. The actual concentrations in the spent solutions measured at the end of the last renewal period were at the level of 72-84% of initial.

The actual concentrations taken from the spend solutions on day 2 and 3 of exposure from 4.6 and 2.2 mg/L, respectively, were at the level of nominal (92-95%).

Based on these results, the average exposure concentrations were calculated (see [Table 2](#)).

**Table 2**  
**Average exposure concentration versus nominal concentration**

MLA-3202 Nominal concentration (mg/L)	Measured concentration (mg/L)				TWA (mg/L)
	t=0h (fresh)	t=24h (old)	t=72h (fresh)	t=96 (old)	
0.22	0.206	0.0918	0.208	0.173	0.164
0.46	0.430	0.189	0.452	0.325	0.334
1.0	0.942	0.664	1.00	0.841	0.854
2.2	1.97	1.7	n.d.	n.d.	1.830
4.6	4.22	3.79	n.d.	n.d.	3.999

n.d. – not determined, no surviving fish were present in this concentration

**7.2.2. Mortality and other effects**

[Table 3](#) shows the mortality data recorded during the final test. No mortality and clinical effects were observed in the control and the two lowest concentrations tested during the 96-h exposure period. The mortality in the concentration of 0.85 mg/L was 43%, while 100% mortality was observed at the two highest concentrations tested. [Table 4](#) specifies the clinical effects observed at different test concentrations.

The responses recorded in this test allowed for reliable determination of an LC<sub>50</sub>.

**Table 3**  
**Incidence of mortality and total mortality during the final test**

MLA-3202 Average exposure concentration (mg/L)	Initial number of fish	Cumulative mortality					Total Mortality (%)
		2¾h	24h	48h	72h	96h	
Control	7	0	0	0	0	0	0
0.16	7	0	0	0	0	0	0
0.33	7	0	0	0	0	0	0
0.85	7	0	0	1	1	3	43
1.8	7	0	0	0	7	7	100
4.0	7	0	0	7	7	7	100

**Table 4**  
**Clinical effects observed during the final test**

MLA-3202 Average exposure concentration (mg/L)	Time of recording (hours)	Specification of effects		Relative number
0.85	72	Loss of equilibrium and swimming at the bottom		4/6
	96	Immobile		4/4
1.8	48	Immobile		7/7
4.0	2¾	Swimming at the bottom		7/7
	24	Haemorrhages		7/7

**7.2.3. Determination of effect concentrations**

[Table 5](#) shows the effect parameters based on average exposure concentrations, see also [APPENDIX 1](#).

**Table 5**  
**Effect parameters**

Effect parameter	0-24 h	0-48 h	0-72 h	0-96 h	
Survival	LC <sub>50</sub>	>4.0	2.5	1.1	0.91
95%-CL	lower	n.d.	1.1	0.72	0.69
	upper	n.d.	5.4	1.7	1.2

CL – confidence limit, n.d. – not determined

### 7.2.4. Experimental conditions

The results of measurement of pH and oxygen concentrations are presented in [Table 6](#) and [Table 7](#). The temperatures measured during the study in the various test vessels are presented in [Table 8](#). All test conditions remained within the ranges prescribed by the study plan (pH: 6.0-8.5, constant within 1 unit; temperature 20-24°C, constant within 2°C; oxygen > 60% of air saturation).

**Table 6**  
**pH-values during the final test**

MLA-3202 Average exposure concentration (mg/L)	Day 0 <sup>1</sup>		Day 1		Day 2		Day 3		Day 4	
	Fresh	Old	Fresh	Old	Fresh	Old	Fresh	Old	n.d.	n.d.
Control	7.9	7.4	7.8	7.5	7.9	7.5	8.0	7.6		
0.16	7.8	7.4	7.7	7.5	7.7	7.5	7.5	7.7		
0.33	7.8	7.4	7.7	7.4	7.8	7.4	7.8	7.6		
0.85	7.8	7.4	7.7	7.5	7.8	7.5	7.8	7.6		
1.8	7.8	7.4	7.7	7.4	7.8	7.9 <sup>2</sup>	n.d.	n.d.		
4.0	7.8	7.4	7.8	7.5 <sup>2</sup>	n.d.	n.d.	n.d.	n.d.		

1. pH of culture medium was: 7.7; 2. Measured during morning assessment when all fish were found dead; n.d. – not determined, no surviving fish were present in this concentration

**Table 7**  
**Dissolved oxygen concentrations (mg/L) during the final test**

MLA-3202 Average exposure concentration (mg/L)	Day 0 <sup>1</sup>		Day 1		Day 2		Day 3		Day 4	
	Fresh	Old	Fresh	Old	Fresh	Old	Fresh	Old	n.d.	n.d.
Control	9.6	6.8	9.5	6.8	9.6	6.7	9.3	6.9		
0.16	9.6	7.0	9.5	7.3	9.5	7.0	9.5	7.4		
0.33	9.5	6.3	9.5	6.6	9.5	6.5	9.5	7.0		
0.85	9.6	6.9	9.5	7.3	9.4	7.4	9.4	7.5		
1.8	9.4	7.0	9.4	6.7	9.4	8.3 <sup>1</sup>	n.d.	n.d.		
4.0	9.5	6.9	9.4	8.2 <sup>1</sup>	n.d.	n.d.	n.d.	n.d.		

1. Measured during morning assessment when all fish were found dead; n.d. – not determined, no surviving fish were present in this concentration

**Table 8**  
**Temperatures (°C) measured during the final test**

MLA-3202 Average exposure concentration (mg/L)	Day 0 <sup>1</sup>		Day 1		Day 2		Day 3		Day 4	
	Fresh	Old	Fresh	Old	Fresh	Old	Fresh	Old	n.d.	n.d.
Control	21	21	21	21	21	21	21	21		
0.16	21	21	21	21	21	21	21	21		
0.33	21	21	21	21	21	21	21	21		
0.85	21	21	21	21	21	20	20	21		
1.8	21	21	21	21	21	21 <sup>2</sup>	n.d.	n.d.		
4.0	21	21	21	21 <sup>2</sup>	n.d.	n.d.	n.d.	n.d.		

1. Temperature of culture medium was: 21°C; 2. Measured during morning assessment when all fish were found dead; n.d. – not determined, no surviving fish were present in this concentration

## 8. CONCLUSION

Under the conditions of the present study, the 96h-LC<sub>50</sub> was 0.91 mg/L based on average exposure concentrations (95% confidence interval between 0.69 and 1.2 mg/L).

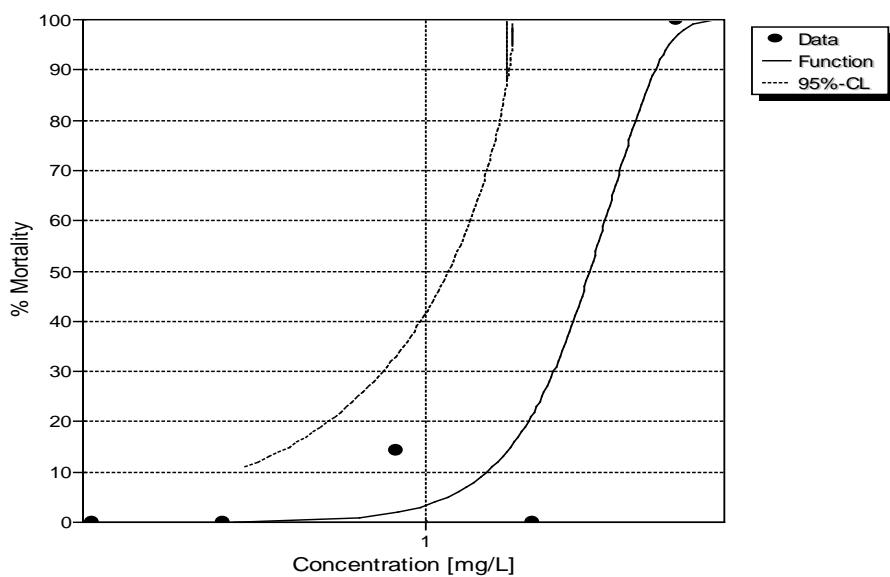
## APPENDIX 1

### EC-VALUES

#### Parameters of the Weibull Analysis at 48h

Parameter	Value
Computation runs:	11.00000
Slope b:	7.67862
Intercept a:	-3.39168
Variance of b:	6.17389
Goodness of Fit	
Chi <sup>2</sup> :	7.74573
Degrees of freedom:	3.00000
p(Chi <sup>2</sup> ):	0.05200
Log LC50:	0.39397
SE Log LC50:	0.06256
g-Criterion:	2.73814
F:	3.69900
p(F) (df: 1;3):	0.15000 <sup>1</sup>

No statistically significant concentration/response was found ( $p(F) > 0.05$ ; i.e. slope of the relationship is not significantly different from zero). Due to the lacking concentration/response the shown LCx are considered indicative.



**Figure 1**  
**Percentage Response (=mortality) as Function of Concentration of MLA-3202 at 48h**

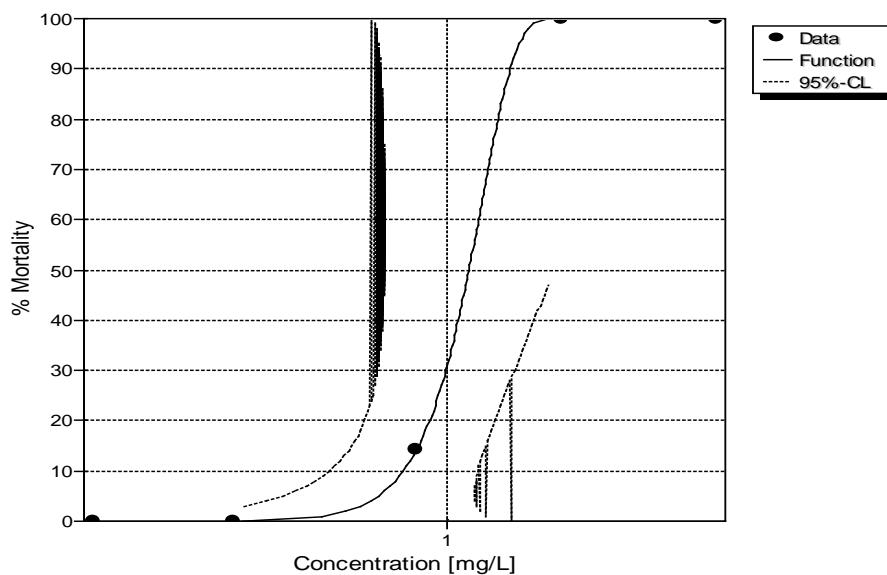
**Table 9**  
**Results of the Weibull Analysis at 48h**

Parameter	EC <sub>50</sub>
Value [mg/L]	2.477
lower 95%-cl	1.138
upper 95%-cl	5.391

**APPENDIX 1**  
**EC-VALUES – continued**

**Parameters of the Weibull Analysis at 72h**

Parameter	Value
Computation runs:	9.00000
Slope b:	12.86525
Intercept a:	-0.97401
Variance of b:	83.92551
Goodness of Fit	
Chi <sup>2</sup> :	0.00472
Degrees of freedom:	3.00000
p(Chi <sup>2</sup> ):	1.00000
Log LC50:	0.04722
SE Log LC50:	0.11110
g-Criterion:	1.94784
F:	1254.51300
p(F) (df: 1;3):	<0.001



**Figure 2**  
**Percentage Response (=mortality) as Function of Concentration of MLA-3202 at 72h**

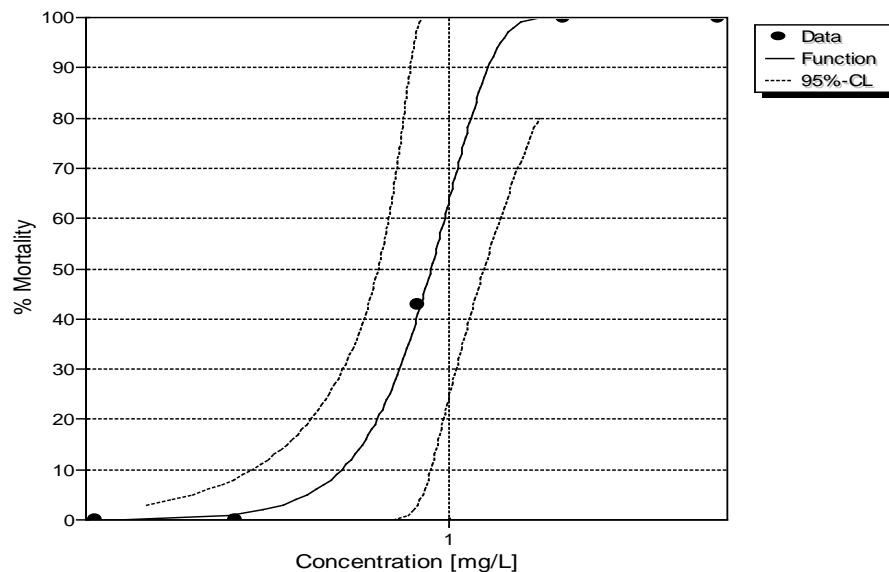
**Table 10**  
**Results of the Weibull Analysis at 72h**

Parameter	EC <sub>50</sub>
Value [mg/L]	1.115
lower 95%-cl	0.720
upper 95%-cl	1.727

**APPENDIX 1**  
**EC-VALUES – continued**

**Parameters of the Probit/Weibull Analysis at 96h**

Parameter	Value
Computation runs:	6.00000
Slope b:	9.37276
Intercept a:	0.02593
Variance of b:	17.91574
Goodness of Fit	
Chi <sup>2</sup> :	0.07292
Degrees of freedom:	3.00000
p(Chi <sup>2</sup> ):	0.99500
Log LC50:	-0.04187
SE Log LC50:	0.06551
g-Criterion:	0.78342
F:	201.73100
p(F) (df: 1;3):	<0.001



**Figure 3**  
**Percentage Response (=mortality) as Function of Concentration of MLA-3202 at 96h**

**Table 11**  
**Results of the Probit/Weibull Analysis at 96h**

Parameter	<b>EC<sub>50</sub></b>
Value [mg/L]	0.908
lower 95%-cl	0.690
upper 95%-cl	1.195

**APPENDIX 2**  
**REFERENCE TEST**

96-hour acute toxicity study in the carp with pentachlorophenol (PCP); Charles River Den Bosch Project 512161 (Batch K15-09)

The study procedure described in this report was based on the OECD guideline No. 203, 1992. In addition, the procedures were designed to meet the test methods of the ISO International Standard 7346-1: Static method, 1996.

Start: 04 January 2016

End: 08 January 2016

This reference test was carried out to check the sensitivity of the test system as used by Charles River Den Bosch. The reference item was pentachlorophenol (PCP, SIGMA, Art. P2604, Batch MKBK1499V).

Concentrations: 0.10, 0.22 and 0.46 mg/l in ISO-medium.

Incidence of mortality observed in the reference study:

Concentration PCP (mg/l) Nominal	Initial Number Of fish	Cumulative number of dead fish recorded at various time points after start of exposure					Total Mortality (%)
		4h	24h	48h	72h	96h	
0.10	5	0	0	0	0	0	0
0.22	5	0	0	1	1	1	20
0.46	5	0	5	5	5	5	100

During the test the pH, oxygen concentration and the temperature of the medium were within the optimal ranges for fish.

Under the conditions of the present test all carp exposed to a PCP concentration of 0.46 mg/l died within 24 hours. One fish died at 0.22 mg/l and no mortality occurred at 0.10 mg/l. The 96h-LC<sub>50</sub> for carp exposed to PCP was 0.24 mg/l. This effect was already reached within 48 hours of exposure.

The range of the 96h-LC<sub>50</sub> for carp is generally between 0.10 and 0.46 mg/l based on historical data of reference tests performed approximately every 3 months from April 1988 until the end of 2000, and annually since then. Hence, the sensitivity of carp originating from the present batch for PCP falls within the range of sensitivities generally observed during the past years.

The study plan, raw data and report of this study are kept in the Charles River Den Bosch archives. The test described above was performed under GLP conditions with a QA-check.

**APPENDIX 3**  
**CERTIFICATE OF ANALYSIS**



Chemtura Corporation  
12 Spencer St  
Naugatuck, CT 06770

Analytical Services  
[www.chemtura.com](http://www.chemtura.com)

**Certificate of Purity**

Customer: Support for Toxicology Studies

Test Substance Name: MLA3202; Amides, tallow, N,N-bis(2-hydroxypropyl)

Physical Appearance: Liquid

CAS No.: 1454803-04-3

Ref. or Lot Number: RC-1045

Date of Analysis: revised March 18, 2016 (original issue March 7, 2016)

Percent Composition	Monoisotopic Mass (daltons)	Formula	Structure/ Identity
33.1	397.4	C <sub>24</sub> H <sub>47</sub> NO <sub>3</sub>	C18:1 (oleic) tallow amides, N,N-bis(2-hydroxypropyl)
22.9	371.3	C <sub>22</sub> H <sub>45</sub> NO <sub>3</sub>	C16:0 (palmitic) tallow amides, N,N-bis(2-hydroxypropyl)
13.6	395.4	C <sub>24</sub> H <sub>45</sub> NO <sub>3</sub>	C18:2 (linoleic) tallow amides, N,N-bis(2-hydroxypropyl)
11.0	399.4	C <sub>24</sub> H <sub>49</sub> NO <sub>3</sub>	C18:0 (stearic) tallow amides, N,N-bis(2-hydroxypropyl)
6.0	369.3	C <sub>22</sub> H <sub>43</sub> NO <sub>3</sub>	C16:1 (palmitoleic) tallow amides, N,N-bis(2-hydroxypropyl)
3.2	419.3	C <sub>26</sub> H <sub>45</sub> NO <sub>3</sub>	C20:4 (eicosatetraenoic) tallow amides, N,N-bis (2-hydroxypropyl)
2.0	393.3	C <sub>24</sub> H <sub>43</sub> NO <sub>3</sub>	C18:3 (linolenic) tallow amides, N,N-bis(2-hydroxypropyl)
1.5	282.3	C <sub>18</sub> H <sub>34</sub> O <sub>2</sub>	C18:1 (oleic) acid
1.1	421.4	C <sub>26</sub> H <sub>47</sub> NO <sub>3</sub>	C20:3 (eicosatrienoic) tallow amides, N,N-bis (2-hydroxypropyl)
5.6			Sum of residual components (< 1% each)
100.0			Total

Blake Lewis 3/7/16  
 Blake Lewis  
 Analytical REACh Scientist, Analytical Services  
 Date  
 Colin Moore 3/7/16  
 Albert J. Nitowski  
 Sr. Technology Manager  
 Analytical and Lab Support Services

**APPENDIX 4**  
**ANALYTICAL REPORT**

**Determination of the  
concentrations**

Author

M.J.C. Brekelmans, MSc.

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**REPORT APPROVAL**



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M.J.C. Brekelmans, MSc.  
Individual Scientist

Date:



## 1. INTRODUCTION

The objective of this analytical study was to determine the actual concentrations in samples taken from the test solutions used during the ecotoxicity test.

For the work detailed in this report, the experimental start date was 05 Dec 2016, and the experimental completion date was 26 Jan 2017.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

Text Table 1  
Chemicals and Reagents

Chemical / Reagent	Supplier
Water	Tap water purified by a Milli-Q water purification system (Millipore, Bedford, MA, USA)
Methanol	Biosolve, Valkenswaard, The Netherlands
Ammonium acetate	Biosolve
ISO-medium	See main report

All chemicals and reagents were of analytical grade, unless specified otherwise.

### 2.2. Methods

#### 2.2.1. Analytical Method

Analysis was based on the analytical method validated for the test item in project 511870.

##### *Analytical conditions*

Instrument	Acquity UPLC system (Waters, Milford, MA, USA)
Detector	Xevo TQ-S mass spectrometer (Waters)
Column	Acquity UPLC HSS Cyano, 100 mm × 2.1 mm i.d., dp = 1.8 µm (Waters)
Column temperature	40°C ± 1°C
Injection volume	5 µL
Mobile phase	10 mM Ammonium acetate in 70/30 (v/v) methanol/water
Flow	0.4 mL/min
MS detection	
Ionisation source	ESI <sup>+</sup>
Cone voltage	50 V
Acquisition	$m/z$ 398.2 → $m/z$ 134 (Collision energy 18 eV) $m/z$ 372.2 → $m/z$ 134 (Collision energy 16 eV) $m/z$ 396.2 → $m/z$ 134 (Collision energy 16 eV) $m/z$ 400.3 → $m/z$ 134 (Collision energy 18 eV)
Quantitation	$m/z$ 398.2 → $m/z$ 134

### **2.2.2. Test Samples**

Test samples were stored in the freezer ( $\leq -15^{\circ}\text{C}$ ). Storage stability of samples under these conditions was demonstrated in project 511870.

On the day of analysis, the test samples were thawed at room temperature. The samples were diluted in a 1:3 (v:v) ratio with methanol and analyzed. If necessary, the samples were further diluted with 75/25 (v/v) methanol/ISO-medium to obtain concentrations within the calibration range.

### **2.2.3. Preparation of Solutions**

#### Stock and Spiking Solutions

Stock solutions of the test item were prepared in methanol at a concentration of 2000 mg/L.

Spiking solutions were made up from a stock solution and/or dilutions of this solution. The solvent of the spiking solutions was methanol.

#### Calibration Solutions

Solutions with the test item in the concentration range of 200 - 30000  $\mu\text{g}/\text{L}$  were prepared in methanol from two stock solutions. The solutions were 100-fold diluted with 75/25 (v/v) methanol/ISO-medium to obtain calibration solutions in the concentration range of 2 - 300  $\mu\text{g}/\text{L}$ .

#### Quality Control (QC) Samples

1 mL blank medium was spiked with the test item at a target concentration of 0.01 or 10 mg/L. The QC samples were treated similarly as the test samples (see paragraph [2.2.2](#) ‘Test Samples’).

Blank QC samples consisting of blank medium were treated similarly to the QC and test samples.

### **2.2.4. Sample Injections**

Calibration solutions were injected in duplicate. Test samples and QC samples were analyzed by single injection.

### 3. CONSTRUCTED VARIABLES

Response (R)

Peak area test item [units]

Calibration curve

$$R = a C_N + b$$

where:

 $C_N$  = nominal concentration [ $\mu\text{g}/\text{L}$ ]a = slope [units  $\times$  L/ $\mu\text{g}$ ]

b = intercept [units]

Analyzed concentration ( $C_A$ )

$$C_A = \frac{(R - b)}{a} \times \frac{d}{1000} \quad [\text{mg}/\text{L}]$$

where:

d = dilution factor

Accuracy

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to nominal concentration

$$\frac{C_A}{C_N} \times 100 \quad [\%]$$

Relative to initial

$$\frac{C_A (t = x \text{ hours})}{C_A (t = 0 \text{ hours})} \times 100 \quad [\%]$$

$$\frac{C_A (t = x \text{ days})}{C_A (t = 0 \text{ days})} \times 100 \quad [\%]$$

Limit of detection (LOD)

$$LOD = \frac{3N}{S} \times C_N$$

where:

 $N$  = noise height [units] $S$  = peak height [units]

### 4. COMPUTERIZED SYSTEMS

Critical computerized systems used in the phase are listed below. All computerized systems used in the conduct of this phase have been validated.

Text Table 2  
Critical Computerized Systems

System name	Version No.	Description of Data Collected and/or Analyzed
MassLynx	4.1	System control, data acquisition and processing
REES Centron	SQL 2.0	Temperature, relative humidity and/or atmospheric pressure monitoring

## 5. RESULTS

### 5.1. Calibration Curves

Calibration curves were constructed using five concentrations. For each concentration, two responses were used. Linear regression analysis was performed using the least squares method with a 1/concentration<sup>2</sup> weighting factor. The coefficient of correlation (r) was > 0.99 for each curve.

### 5.2. Samples

#### 5.2.1. QC Samples

The results for the QC samples are given in [Table 1](#).

A small response at the retention time of the test item was detected in the chromatograms of the blank QC samples. Concentration was below the limit of detection which was 0.00029 mg/L during the range-finding test and 0.00012 mg/L during the final test.

During the range-finding test, the mean accuracy of the 0.01 mg/L QC samples was slightly above the criterion range of 70-110% (i.e. 111% of target). The mean accuracy of the 10 mg/L QC samples was within the criterion range. During the final test, the mean accuracies of QC samples containing test item fell within the criterion of 70-110%. It demonstrated that the analytical method was adequate for the determination of the test item concentration in the test samples.

#### 5.2.2. Test Samples

The results for the test samples are given in [Table 2](#) and [Table 3](#).

**Table 1**  
**QC Samples**

Date of preparation	Date of analysis	Concentration [mg/L]			Accuracy [%]	
		Target	Nominal	Analyzed	Individual	Mean
05 Dec 2016	05 Dec 2016	0	0.00	< LOD	n.a.	n.a.
			0.00	< LOD	n.a.	
05 Dec 2016	05 Dec 2016	0.01	0.0100	0.0114	114	111
			0.0100	0.0107	107	
05 Dec 2016	05 Dec 2016	10	10.0	9.42	94	99
			10.0	10.3	103	
26 Jan 2017	26 Jan 2017	0	0.00	< LOD	n.a.	n.a.
			0.00	< LOD	n.a.	
26 Jan 2017	26 Jan 2017	0.01	0.0100	0.0102	102	102
			0.0100	0.0102	102	
26 Jan 2017	26 Jan 2017	10	10.0	9.87	99	100
			10.0	10.0	100	

LOD The limit of detection of the method, taking a dilution factor of four into account, was determined to be 0.00029 mg/L on 05 Dec 2016 and 0.00012 mg/L on 26 Jan 2017.

n.a. Not applicable.

**Table 2**  
**Range-finding Test: Test Samples**

Time of sampling [hours]	Date of sampling	Date of analysis <sup>1</sup>	Concentration [mg/L]		Relative to nominal [%]	Relative to initial [%]
			Nominal	Analyzed		
0	21 Nov 2016	05 Dec 2016	0.1	0.102	102	
			1.0	1.12	112	
			10	10.7	107	
24	22 Nov 2016	05 Dec 2016	0.1	0.0612	61	60
			1.0	0.967	97	87
			10	10.4	104	98
96	25 Nov 2016	05 Dec 2016	0.1	< LOD		
			1.0	0.0038 <sup>2</sup>	0.38	0.34
			10	12.0	120	113

<sup>1</sup> Samples were stored in the freezer ( $\leq -15^{\circ}\text{C}$ ) until the day of analysis.<sup>2</sup> Estimated value, calculated by extrapolation of the calibration curve.

LOD The limit of detection of the method, taking a dilution factor of four into account, was determined to be 0.00029 mg/L.

**Table 3**  
**Final Test: Test Samples**

Time of sampling [days]	Time of sampling [hours]	Date of sampling	Date of analysis <sup>1</sup>	Concentration [mg/L]		Relative to nominal [%]	Relative to initial [%]
				Nominal	Analyzed		
0 (fresh)	0 (fresh)	16 Jan 2017	26 Jan 2017	0	< LOD		
				0.22	0.206	93	
				0.46	0.430	93	
				1.0	0.942	94	
				2.2	1.97	89	
				4.6	4.22	92	
1 (old)	24 (old)	17 Jan 2017	26 Jan 2017	0	< LOD		
				0.22	0.0918	42	45
				0.46	0.189	41	44
				1.0	0.664	66	70
				2.2	1.70	77	86
				4.6	3.79	82	90
2 (old)	24 (old)	18 Jan 2017	26 Jan 2017	4.6	4.21	92	
3 (old)	21 (old)	19 Jan 2017	26 Jan 2017	2.2	2.09	95	
3 (fresh)	0 (fresh)	19 Jan 2017	26 Jan 2017	0	< LOD		
				0.22	0.208	94	
				0.46	0.452	98	
				1.0	1.00	100	
4 (old)	24 (old)	20 Jan 2017	26 Jan 2017	0	< LOD		
				0.22	0.173	78	83
				0.46	0.325	71	72
				1.0	0.841	84	84

<sup>1</sup> Samples were stored in the freezer ( $\leq -15^{\circ}\text{C}$ ) until the day of analysis.

LOD The limit of detection of the method, taking a dilution factor of four into account, was determined to be 0.00012 mg/L.